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ARTICLE

Targeted disruption of the mouse sphingolipid activator protein gene: a complex phenotype, including severe leukodystrophy and wide-spread storage of multiple sphingolipids

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The four established or putative sphingolipid activator proteins derive from a large precursor protein encoded by a single gene. In addition to generating the four sphingolipid activator proteins, the precursor protein is suspected of having functions of its own, as, for example, a lipid binding/transport protein or a neurotrophic factor. The gene also appears to encode the Sertoli cell major sulfated glycoprotein. Sequence similarities have been noted with many other proteins of diverse functions. One patient and a fetus in a single family with a complete defect of this gene due to a mutation in the initiation codon exhibited complex pathological and biochemical abnormalities. Mutant mice homozygous for an inactivated gene of the sphingolipid activator protein precursor exhibit two distinct clinical phenotypes—neonatally fatal and later-onset. The latter develop rapidly progressive neurological signs around 20 days and die by 35–38 days. At 30 days, severe hypomyelination and periodic acid-Schiff-positive materials throughout the nervous system and in abnormal cells in the liver and spleen are the main pathology. Most prominently lactosylceramide, and additionally ceramide, glucosylceramide, galactosylceramide, sulfatide, and globotriaosylceramide are abnormally increased in the brain, liver, kidney, and their catabolism abnormally slow in cultured fibroblasts. Brain gangliosides are generally increased, particularly the monosialogangliosides. The clinical, pathological and biochemical phenotype closely resembles that of the human disease. This model not only allows further clarification of the physiological functions of the four individual sphingolipid activator proteins but also should be useful to explore putative functions of the precursor protein.

INTRODUCTION

Sphingolipid activator proteins (SAPs) are small lysosomal glycoproteins which function as essential cofactors for physiological degradation of sphingoglycolipids with relatively short hydrophilic head groups (1). The GM2-activator protein stimulates degradation of GM2-ganglioside and asialo GM2-ganglioside (GA2) by β-hexosaminidase A and is encoded by a gene on human chromosome 5 (2–4). Four other known sphingolipid activator proteins ('*saps*', 'saposins'; see ref. 5 for various nomenclature systems) are homologous to each other and are encoded tandemly by a single gene localized on human chromosome 10 (6–9). Among them, SAP-B and SAP-C had been known for decades as the sulfatide activator (10) and the glucosylceramide activator (11), respectively, although their genetic relationship was not recognized until several years ago (6,7,12). The two

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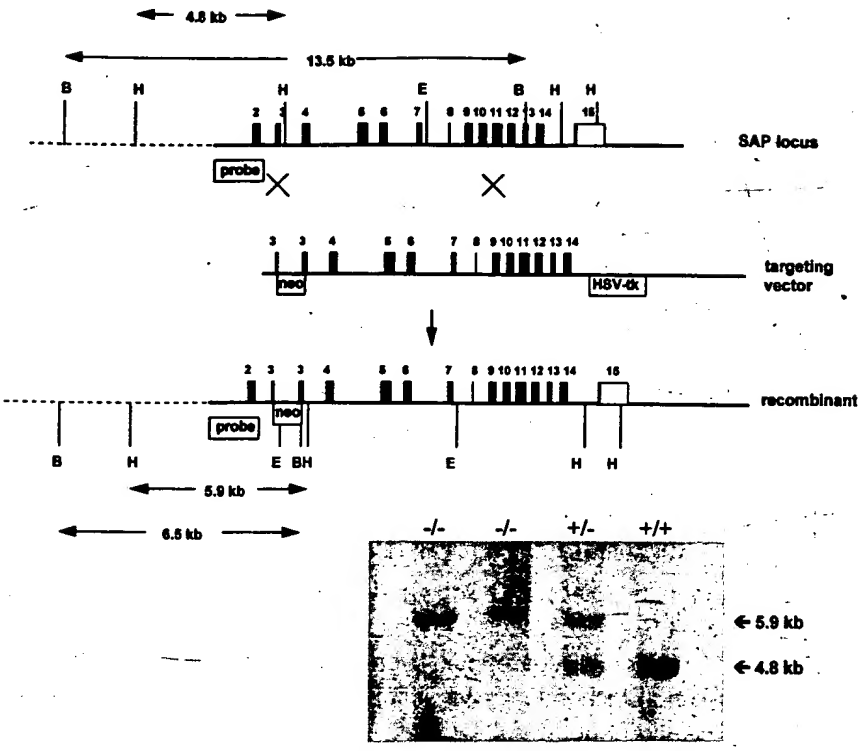


Figure 1. The targeting strategy and the Southern analysis of the mouse genotype. For double selection, the neomycin-resistant gene is inserted within exon 3 and the HSV-tk gene is inserted at the *SalI* site in the 3′ region. The constructed targeting vector was linearized by *NotI* digestion. The size of the regions for homologous recombination was 0.7 kb and 8.5 kb, respectively, on the upstream and downstream sides of the neo gene. The wild-type and recombinant genes can be distinguished by either *Hin*dIII or *Bam*HI digestion and Southern analysis, which shows the patterns of the *Hin*dIII restriction fragments in the three different genotypes. The location and approximate size of the probe is indicated on the map. B: *Bam*HI, H: *Hin*dIII, E: *Eco*RI.

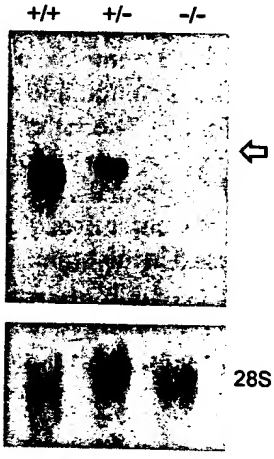
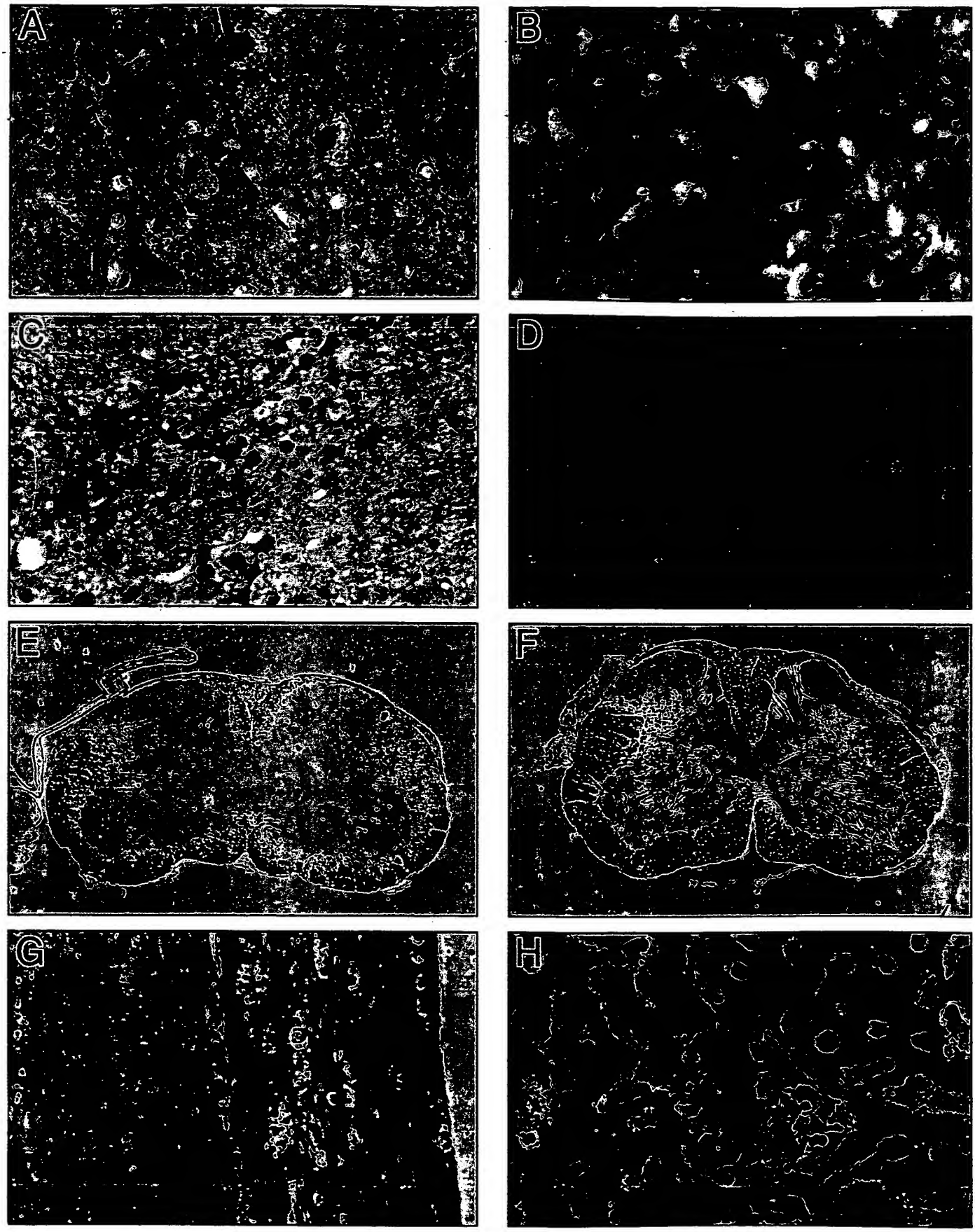


Figure 2. Northern analysis of the SAP mRNA in the three genotypes. No recombinant SAP mRNA can be detected in the SAP –/– mouse. The arrow indicates the expected position of the recombinant mRNA, if the recombinant gene generated a stable, processed transcript. It should be approximately 1 kb larger than the normal mRNA.

additional flanking homologous domains within the same transcript, SAP-A and SAP-D, were discovered also only as the consequence of the cDNA cloning. In restrospect, Wenger had observed a disproportionately large precursor protein in his studies of SAP-B and SAP-C (13,14). Degradation of many natural as well as chemically modified sphingolipids is stimulated by one or more of these SAPs *in vitro* (1,15). However, physiologically meaningful activating functions have been established for a relatively few sphingolipids through either experiments in living cultured cells or existence of disorders due to genetic deficiencies of these small glycoproteins. If one relies only on the data observed in living cells and/or organisms, SAP-B is a natural activator for degradation of sulfatide and globotriaosylceramide, SAP-C for glucosylceramide, and SAP-D for ceramide. The physiological activator function for SAP-A has not been established under these criteria. Genetic deficiencies of SAP-B and SAP-C in man result in disorders mimicking metachromatic leukodystrophy and Gaucher disease, respectively, and several disease-causing mutations have been identified in the SAP-B domain (16–21) and in the SAP-C domain (22,23). Two sibs in a consanguineous family, affected by a rapidly fatal disorder with highly complex clinical, pathological and bio-

Figure 3. All sections are from paraffin-embedded specimens, except for B, which is a frozen section. (**A**) SAP –/– cerebral cortex stained with luxol fast blue and PAS; (**B**) SAP –/– frozen section of cerebral cortex stained with luxol fast blue and PAS. Cerebral cortical neurons contain storage materials that stain strongly positive with PAS in frozen sections (B) but not in paraffin sections (A). (**C**) SAP –/– cerebral white matter stained with luxol fast blue and PAS, showing marked paucity of myelin and presence of numerous axonal spheroids that stain lightly positive with PAS. (**D**) Spinal white matter, stained with luxol fast blue and PAS. Myelination is relatively more advanced in the spinal cord than in the cerebral white matter. Degenerating myelin and scattered PAS positive macrophages are present. Cross sections of the spinal cord of SAP –/– (**E**) and control mice (**F**) stained histochemically with an antibody against myelin basic protein. (**G**) SAP –/– spinal root, stained with solochrome and eosin where myelin degeneration is also evident. (**H**) SAP –/– liver stained with PAS. Clustered histiocytes/macrophages are conspicuous in the hepatic sinusoid.



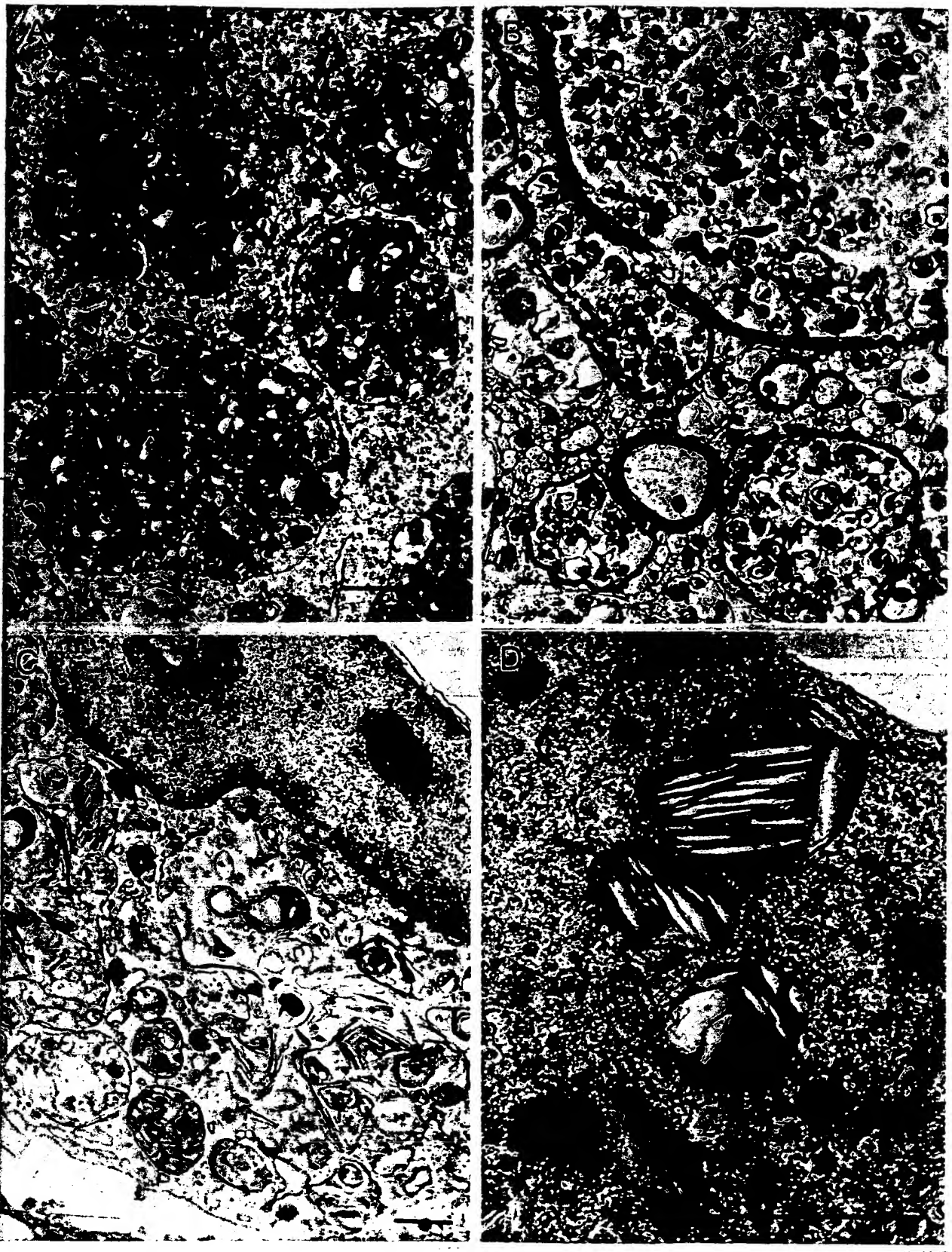


Figure 4. Electron micrographs of neuronal inclusions (**A**), axonal spheroids (**B**). Inclusions in a sinusoidal cell (**C**) and inclusions in a hepatocyte (**D**). The lines indicate the scale of 1 μm.

chemical abnormalities, were found to be homoallelic for a mutation in the initiation codon of the SAP gene (24–29). None of the SAPs were detected in the tissues, and liver cells and cultured fibroblasts showed a dramatically altered organization of the endosomal/lysosomal system characterized by accumulation of multivesicular bodies. Abnormal accumulation of multiple sphingolipids were noted in the brain and systemic organs.

The products of the sphingolipid activator gene appear to have other important physiological functions in addition to the well-established functions as sphingolipid activators. The same gene may also code for the major sulfated glycoprotein of the Sertoli cell which is postulated to be essential in the process of spermatogenesis (30). Recently, putative functions for the SAP precursor protein itself have been proposed, including functions as a ganglioside binding/transport protein (31), a neurotrophic factor (32,33), and possible roles in gonadal development (34). In addition, the primary amino acid sequences of the SAPs and their precursor protein show significant similarities, if not homology, to many other seemingly unrelated proteins, including the lung surfactant-associated protein B (35), suggesting possible common mechanisms underlying their functions (36).

We have generated a mutant mouse line in which the sphingolipid activator protein gene has been inactivated by the homologous recombination technology in order to explore the diverse functions of the precursor and processed products of the sphingolipid activator protein gene. Affected mice show highly complex clinical, pathological and biochemical abnormalities similar to the human patients with total SAP deficiency. This article provides the basic characterization of this model which should be useful not only for further clarification of the multiple functions of the products of this unusually complex gene but also for gene therapy trials.

RESULTS

Molecular genetic analysis

The SAP –/– line of mice was generated from one of the targeted Es cell lines, S29. The Southern blot analysis of genomic DNA fragments, generated by either *Hin*dIII or *Bam*HI digestion, gave the size distribution expected from the restriction maps of the normal and targeted SAP gene (Fig. 1). On the Northern blot analysis, no SAP precursor mRNA could be detected from tissues of affected homozygous mice, while its quantity was reduced in heterozygous carriers, as expected (Fig. 2).

Clinical phenotype

Of the first 126 offspring from carrier-to-carrier mating that survived beyond a few days after birth, 36 (29%) mice had the wild-type genotype, 68 (55%) mice were carriers and 16 (13%) were homozygous affected. Genotype determination was inconclusive for three mice. Five of the total nine mice that died within a day or two of birth were SAP –/–. Therefore, a disproportionate percentage of SAP –/– mice appear to die *in utero* or during the neonatal period. Those SAP –/– mice that survive beyond the first few days feed and grow normally together with their littermates until about 18–20 days when they develop first neurological symptoms. By then, affected mice are slightly smaller than their littermates. Initial symptoms are tremulousness of the head and mild weakness/ataxia of the hind legs. These manifestations progress rapidly during the next ten days to gross shaking of the head and then the trunk, and severe weakness of all legs. After 30 days, intermittent seizures develop and progress to continual tonic status epilepticus. Affected mice die around 35 days in an emaciated general condition. Attempts at keeping affected mice alive beyond 35–38 days by forced feeding have been unsuccessful. By then, the body size of the affected mice is less than 1/2 of their littermates.

Histopathology

The external aspects of the brains of SAP –/– mice appeared grossly normal. However, on coronal sections, the white matter of SAP –/– mouse was pale and ill-defined from the gray matter, suggesting paucity of myelin. No gross abnormalities were noted in the visceral organs. There was no organomegaly. The size of the kidney was significantly smaller in the affected mice (207 ± 31 mg/2 kidneys, $n = 4$) than in the control mice (253 ± 34 mg/2 kidneys, $n = 5$), consistent with the smaller bodies of the affected mice.

The primary neuropathology of SAP –/– mouse was that of combined neuronal storage and leukodystrophy. No obvious abnormalities in brain development or the cellular migration and architecture of the central nervous system were noted on the light microscopic level, although detailed morphometric studies have not yet been undertaken. There was neuronal storage in the retinal ganglion cells and in the neurons throughout the brain and spinal cord. The storage material was periodic acid-Schiff (PAS)-negative on paraffin sections and appeared as finely vacuolated (Fig. 3A) or as coarse eosinophilic granules. However, they were strongly PAS-positive in frozen sections (Fig. 3B). Finely vacuolated storage neurons were more conspicuous in the cerebral cortex while eosinophilic storage materials were more prominent in the neurons in the entorhinal cortex. In the hippocampus, storage neurons were most abundant in the CA3 region. There were many spherical or ovoid structures containing eosinophilic granular materials, some of which were Bielschowsky-positive. They are most likely distended axons (axonal spheroids) (Fig. 3C). Scattered PAS-positive cells with small round nuclei identified as macrophages/microglia were present in the white matter (Fig. 3D), basal ganglia, thalamic nuclei, the cerebellar molecular layer as well as in some areas of the cerebral cortex. The white matter was generally hypomyelinated, most prominently in the corpus callosum and cerebral white matter and least in the spinal cord (Fig. 3E, F). Myelin ovoids suggesting degeneration of myelin were also noted in the corpus callosum, the internal capsule and the spinal white matter. Spinal roots, and the trigeminal and sciatic nerves showed similar degenerative changes of myelin (Fig. 3G), although the extent of degeneration was variable. In the cerebellum, PAS-positive macrophages/microglia were noted in the molecular layer as well as in the white matter. Neuronal storage was present in the deep cerebellar nuclei but not apparent in the Purkinje or granular cells, although the soma of some Purkinje cells was vacuolated. Many Bielschowsky stain-positive axonal spheroids were present in the cerebellar white matter. The neuronal storage was also noted in the trigeminal and spinal dorsal root ganglia. Neuronal storage and myelin degeneration were also present in the spinal cord. No sudanophilic or metachromatic materials were found in any of the sections examined.

In the liver, clusters of histiocytes with abundant eosinophilic cytoplasm were seen in the hepatic sinusoid. These cells were only faintly PAS-positive, while hepatocytes were strongly PAS-positive even in paraffin sections (Fig. 3H). Similar cells

were noted in the spleen and lymph nodes. The kidney, heart and lungs appeared normal on the light microscopic level.

Electron microscopic studies revealed that the storage materials in the cerebral cortical neurons consisted of scattered or variously clustered small concentric lamellar and/or electron-dense granular structures. They were often bound by a membrane (Fig. 4A). Distended axons (axonal spheroids) contained similar concentric or lamellar structures, which, however, were not clustered (Fig. 4B). Ultrastructural features of the intracellular storage materials in the hepatic sinusoids were more complex than those in neurons. They were composed of concentric outer membranes and short straight or curved membranous or small vesicular inner structures (Fig. 4C). Some hepatocytes contained electron-dense membrane-bound inclusions, which displayed electronlucent short needle-like inner structures (Fig. 4D).

Biochemistry

Brain lipids (Fig. 5A; Table 1). The most conspicuous specific abnormality in both gray and white matter was a major accumulation of lactosylceramide (more than 50-fold normal), which appeared to account for the strong PAS staining on frozen sections. The chromatographic behavior of lactosylceramide suggested an identical fatty acid composition in gray and white matter. A small but clearly pathological amount (estimated at about 30–50 nmol/g) of another glycolipid migrating as gangliotriaosylceramide was also detected. Due to the small size of the brains analyzed, no attempt was made to quantitate glucosylceramide. By visual inspection of appropriate thin-layer chromatograms, no clear increase of free ceramide could be found in the brain. From visual inspection of the total lipid fraction as well as the quantitative data, the white matter lipids reflected the lack of myelin observed histologically, with reductions of both galactosylceramide and sulfatide to approximately 25% of the normal amounts, together with diminished amounts of cholesterol and phospholipids, particularly ethanolamine phospholipids and sphingomyelin with longer-chain fatty acids. No obvious abnormalities in the levels of cholesterol, glycerophospholipids and sphingomyelin were observed in the gray matter.

The total ganglioside sialic acid was increased significantly in both gray and white matter, more conspicuously in the latter with large relative increases in the monosialogangliosides, GM1, GM2 and GM3 (Fig. 5B). Their relative increase alone can be interpreted as non-specific. However, because of the substantial increases in the total ganglioside sialic acid, some of the higher gangliosides, most notably GD1a, were also significantly increased on the wet weight or total lipid basis. This finding is unusual and to our knowledge has been described only in an unusual genetic disease occurring in the emu (37).

Liver and kidney lipids (Fig. 6; Table 1). There were multiple, complex abnormalities in the sphingolipid composition in these organs. Similar to the brain, lactosylceramide was conspicuously increased in both organs (Fig. 6A,B), more prominently in the liver (approximately 50 times normal) than in the kidney. In addition, ceramide, glucosylceramide, globotriaosyl-ceramide, and globoside were all significantly increased in the liver. All of these lipids were present at or below detectability in normal liver with the scale of analysis employed. These lipids were also increased in the kidney (data not shown). However, the major attention in the kidney was focused on glucosylceramide, galactosylceramide and sulfatide. Because of the very small quantities of these lipids in the kidney, data were obtained on pooled samples and the results expressed as the ratio to the control kidneys. Galactosylceramide, glucosylceramide and sulfatide were all increased approximately 5-fold over the control kidneys. Lactosylceramide sulfate was also mildly elevated. Thus, in addition to the increased glucosylceramide, both of the myelin-characteristic galactolipids, galactosylceramide and sulfatide, were also significantly increased in the kidney of the total SAP-deficient mice, despite the secondary decrease in these lipids in the brain due to the lack of myelin. There were no significant abnormalities in cholesterol, glycerophospholipids and sphingomyelin in the liver and kidney.

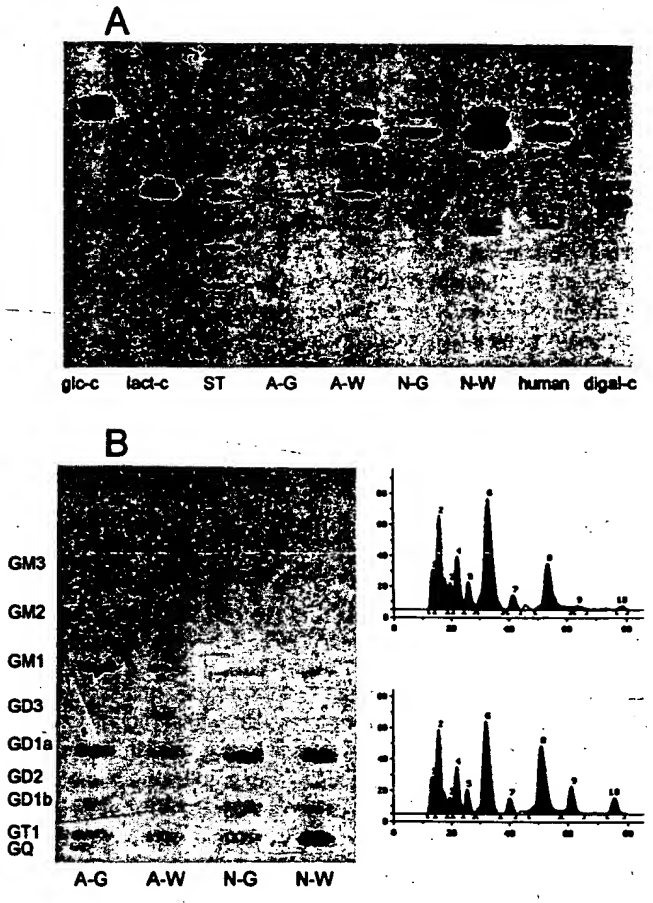


Figure 5. (**A**) Thin-layer chromatogram of brain sphingolipids. The brain lipid extract was subjected to the mercuric chloride/saponification procedure and non-polar lipids removed by column chromatography. Solvent system: chloroform-methanol-water (65:25:4, v/v/v). Visualization: orcinol spray. Lipids corresponding to 5 mg of starting tissue were applied for affected gray (AG), affected white (AW), normal gray (NG) and normal white matter (NW). The band corresponding to lactosylceramide is conspicuous in the affected brain. Galactosylceramide (faster-moving doublet) and sulfatide (slower-moving doublet) are decreased in the SAP –/– brain. Other lanes are standard lipid samples. ST: a mixture of lactosylceramide (doublet due to fatty acid composition), globotriaosylceramide and globoside. Human: similarly processed normal human brain sphingolipid mixture. (**B**) Thin-layer chromatogram and scans of brain ganglioside. The ganglioside fraction was applied at 20 nmol of sialic acid. AG: affected gray, AW: affected white, NG: normal gray, NW: normal white. The plate was run in the solvent system of chloroform-methanol-0.2% $CaCl_2$ (55:45:10, v/v/v) and spots visualized by the resorcinol spray and heating. The upper scan is from the normal brain and the lower scan from SAP –/–. Relative increases in the three monosialogangliosides, GM1, GM2 and GM3, are evident.

Table 1. Quantitative analyses of tissue lipids

	Liver		Brain			
			Gray matter		White matter	
	Affected	Controls	Affected	Controls	Affected	Controls
Total-lipid P	33.6 ± 1 .9	36.4 ± 2.5	55.8 ± 3.4	49.9 ± 4.1	60.2 ± 2.5	69.4 ± 6.8
Sphingomyelin[a]	2.5	1.9	2.7	3.0	3.6	4.2
Total cholesterol	14.6 ± 1 .1	12.8 ± 1 .4	45.9 ± 1.7	41.6 ± 2.9	51.6 ± 3.2	73.2 ± 12.3
Total lipid NeuAc			5.88 ± 0.51	3.65 ± 0.12	5.70 ± 0.73	2.86 ± 0.46
Galactosylceramide[a]					6.6	20.0
Glucosylceramide[a]	0.45	<0.03				
Lactosylceramide[a]	0.75	<0.03	0.35	<0.01	0.50	0.01
GbOse3Cer[a,b]	0.10	<0.02				

Concentrations are expressed as μmol/g wet weight. The numbers of the samples, when analyzed separately, were 4 for the affected and 5 for the controls.
[a]Performed on pooled samples.
[b]Globotriaosylceramide.

Lysosomal enzymes. Activities of the lysosomal enzymes that might be relevant to the sphingolipid activator proteins were assayed either with chromogenic or fluorogenic substrates or with natural lipid substrates under the optimal *in vitro* conditions in the presence of suitable detergents. Liver samples, rather than the brain, were used for this purpose in order to avoid the complications from the secondary changes due to the severe neuropathology. The results showed significant differences between the SAP –/– and controls in some enzymes (Table 2). The most noteworthy was the activity of glucosylceramidase which was 25% of the controls, either assayed with 4-methylumbelliferyl β-glucoside under a specific condition to measure the glucosylceramidase activity or with the natural lipid substrate. Activity of galactosylceramidase in SAP –/– mice was approximately 50%, and that of sphingomyelinase approximately 60% of the controls. On the other hand, acid β-galactosidase (GM1-ganglioside β-galactosidase) and arylsulfatase A activities were unchanged, while α-galactosidase and total β-hexosaminidase were moderately elevated in the SAP –/– mice.

Table 2. Lysosomal enzyme activities in the liver

Enzymes	Affected (n = 4)	Controls (n = 5)
	(nmol/h/mg protein)	
β-Galactosidase	108 ± 47	96 ± 41
Galactosylceramidase	0.88 ± 0.22	1.58 ± 0.32
β-Glucosidase[a]	7.2 ± 1.2	28.8 ± 5.4
Glucosylceramidase	9.5 ± 1.0	41.0 ± 4.7
Sphingomyelinase	27.1 ± 8.3	44.7 ± 4.8
Arylsulfatase A	21 ± 4	23 ± 3
α-Galactosidase	32 ± 6	22 ± 3
Total β-hexosaminidase	1 520 ± 116	1 020 ± 118

[a]The assay system included sodium taurocholate and these values represent the β-glucosidase activities due to glucosylceramidase.

Metabolic studies with cultured fibroblasts (Fig. 7). These experiments were done with skin fibroblasts obtained from 30 day old mice. The results essentially corroborated the analytical findings on various organs described above. Increased retention of the radioactive label was found in ceramide, glucosylceramide and lactosylceramide. In addition, GM3-ganglioside retained more radioactivity than the control fibroblasts. Most importantly, the general pattern of sphingolipid abnormalities in the cultured mouse fibroblasts was qualitatively identical with that of the cultured fibroblasts from the human patients with genetic total SAP deficiency.

DISCUSSION

The Northern blot analysis demonstrated complete absence of SAP mRNA in affected homozygous mice. This finding was particularly important because of the nature of the SAP gene. The targeted SAP gene was interrupted at exon 3. Ordinarily, this would have assured complete loss of the functional translation product. However, the normal SAP mRNA is translated and then processed to generate four small sphingolipid activator proteins. Exon 3 is within the domain of SAP-A and there are several methionine codons downstream of the disrupted site. If the transcript could be processed to a stable mRNA and if any of these downstream ATG could serve as the initiation codon, there is a possibility that a precursor protein truncated at the *N*-terminus might be synthesized and subsequently processed to generate some of the downstream SAPs with normal sequences. The complete absence of mRNA excludes such a possibility.

Two clinical forms, the *in utero*/neonatally fatal form and the dysmyelinating form, were observed among the homozygous affected mice. Genotype of surviving mice suggests that up to half of SAP –/– mice may die *in utero* or within the first 1–2 days after birth. Mice with the neonatal form are not included in this initial characterization because of the complexity of the main, later-onset phenotype. The present report concentrates on the brain, liver and kidney of the main later-onset phenotype, specifically at 30 days, for the sake of consistency. The neonatally fatal form, ontogeny of pathology and biochemical abnormalities of the main phenotype, and other organ systems, such as testis, will be studied in detail in the next phase of exploration of this mutant mouse.

The main neuropathological findings of SAP –/– mice at 30 days are presence of numerous dystrophic axons, extensive

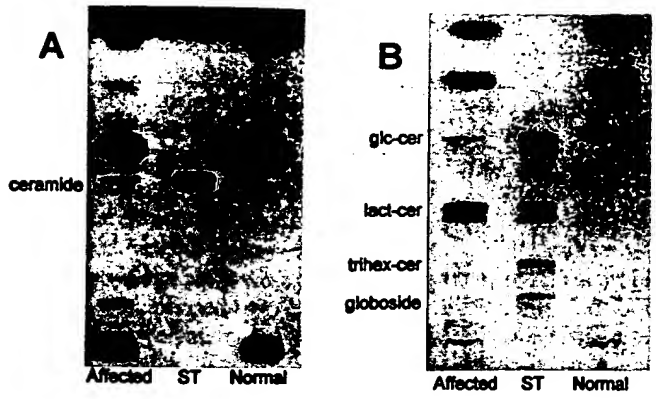


Figure 6. Thin-layer chromatograms of liver sphingolipids after saponification and removal of polar lipids. The same lipid fraction corresponding to 5 mg of starting tissue was run in chloroform-methanol-acetic acid (47:1:2, v/v/) (**A**) and sequentially in chloroform-methanol-water (65:25:4, v/v/v) followed by hexane-ethylacetate-acetic acid (90:10:1, v/v/v) (**B**) and visualized by copper acetate/phosphoric acid spray and heating. Accumulations of ceramide, lactosylceramide, globotriaosylceramide ('trihex-cer') and globoside are demonstrated. The most striking is the degree of lactosylceramide accumulation. On the scale of analysis employed, these sphingolipids, with the exception of ceramide, are essentially undetectable in normal liver.

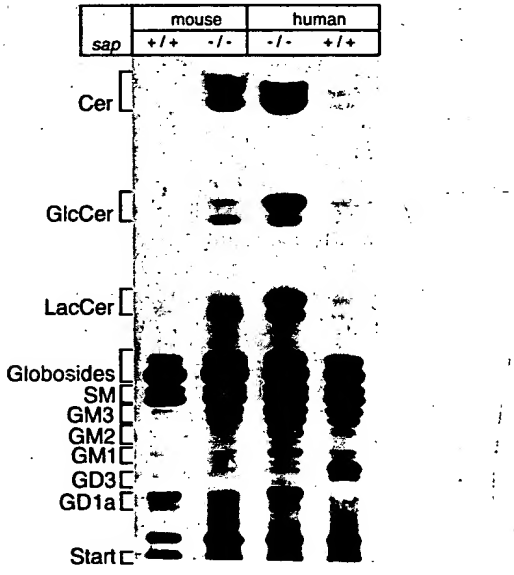


Figure 7. Turnover of labeled sphingolipids in cultured fibroblasts from affected mice in comparison to those from human total SAP-deficiency fibroblasts. Fibroblasts from a 30 day old affected mouse (mouse SAP –/–), fibroblasts from a patient with SAP-precursor-deficiency who died at 16 weeks (human SAP –/–) and their respective controls are compared. The methodological details are described in Materials and Methods. The entire samples were chromatographed. The protein contents of the samples were 141 µg (mouse +/+), 157 µg (mouse –/–), 203 µg (human –/–) and 133 µg (human +/+). Thus the results from the two mouse samples are comparable but the differences between the human patient and control are quantitatively exaggerated.

neuronal storage and severe leukodystrophy. The latter changes include hypomyelination as well as myelin degeneration. Dystrophic axons and hypomyelination have been well documented in both human and murine Niemann-Pick disease type C (NPC) (38,39). However, the ultrastructural features of storage cells in the visceral organs as well as in the neurons of SAP –/– mice differ from those of the NPC. The ultrastructural morphology of the neuronal storage materials in SAP –/– mice also differ significantly from those of known neuronal storage diseases in humans, such as gangliosidoses (40), mucopolysaccharidoses (40), neuronal ceroid lipofuscinosis (41), or in the β-hexosaminidase α- or β-subunit knockout mice (42–44). The neuronal inclusions in SAP –/– mice have some resemblance to those in cultured skin fibroblasts from the human patients with total SAP deficiency (24,28), although neuronal inclusions in these patients have not been recorded in the literature. In the total SAP-deficient patient, hepatic sinusoidal cells contained clusters of membrane-bound vesicles, which had some similarity to the inclusions found in these cells in the SAP–/– mice. In addition, inclusions resembling 'Gaucher disease body' were described in sinusoidal cells in the SAP –/– patient (24) but such inclusions were not detected in our SAP –/– mice. None of the pathological or biochemical findings can readily explain the pathogenetic mechanism for the severe leukodystrophy.

Patients with specific SAP-B deficiency show a general clinical phenotype resembling metachromatic leukodystrophy caused by genetic arylsulfatase A deficiency, because SAP-B is primarily the sulfatide activator *in vivo* (45–48). One patient was reported to have neuropathological features of metachromatic leukodystrophy with storage of brown metachromatic materials in neurons, glial cells and macrophages (49). Despite our specific effort to find metachromatic materials in the SAP –/– mouse, they were not found either in the nervous system or in visceral organs.

The biochemical abnormalities are complex but largely consistent with the known *in vivo* specificities of the individual SAPs, A, B, C and D. The *in vivo* function of SAP-A has not been established but there are suggestive pieces of *in vitro* observation that it might be a galactosylceramidase activator (50). Sulfatide and globotriaosylceramide are the only established substrates, degradation of which is stimulated *in vivo* by SAP-B (1). The only established enzyme SAP-C stimulates *in vivo* is glucosylceramidase (1), while the only definitive *in vivo* function of SAP-D is activation of ceramide degradation by ceramidase (51,52). All of these sphingolipid substrates were abnormally increased in the tissues analyzed. The major accumulation of lactosylceramide is of interest. Both galactosylceramidase and the other β-galactosidase (GM1-ganglioside β-galactosidase, 'acid β-galactosidase') can hydrolyze lactosylceramide under certain specific *in vitro* conditions in the presence of detergents (53). Zschoche *et al.* (54) demonstrated in a liposome system that SAP-C could specifically stimulate hydrolysis of lactosylceramide by galactosylceramidase and that SAP-B could in turn stimulate hydrolysis of lactosylceramide by acid β-galactosidase (54). These observations suggest that, while lactosylceramide can be hydrolyzed by either of the two lysosomal β-galactosidases, each enzyme requires its own specific activator protein *in vivo*, SAP-C for galactosylceramidase, and SAP-B for acid β-galactosidase. Because of the two genetically distinct lysosomal β-galactosidases being active on lactosylceramide, we had earlier predicted that there will be no genetic disease in which lactosylceramide will be the specific abnormal storage compound. However, since both of the activator proteins essential for degradation of lactosylceramide are encoded by a single gene, disruption of the SAP gene will result in a genetic condition in

which this lipid accumulates, although lactosylceramide is only one of many abnormal storage compounds. In some disorders, such as GM1- and GM2-gangliosidoses, there is a mild accumulation of lactosylceramide (55). However, the degree of its increase in the brain of these mice was the highest we have experienced in any disease, human or animal models. The increase in the lactosylceramide in the affected mouse brain was similar to or higher than that in the gray matter of patients with Niemann-Pick type C disease (56–59) as well as that in the spontaneous mouse mutant equivalent to human Niemann-Pick type C disease (60,61), the disease with the highest increase in brain lactosylceramide to our knowledge. In fact, the patient originally described as having 'lactosylceramidosis' (62) later turned out to have Niemann-Pick type C disease (63,64). The lactosylceramide concentration in human Niemann-Pick type C disease brain is approximately 0.3 μmol/g (56) compared to 0.35–0.5 μmol/g in our mouse. The conspicuous PAS-positive materials in the brain, demonstrable only on frozen sections, is likely to be the pathological counterpart of this lactosylceramide accumulation. Lactosylceramide was also the major storage lipid in the human patient with total SAP deficiency (24,28). The collective findings in the liver and kidney indicate that ceramide, glucosylceramide, galactosylceramide, lactosylceramide, sulfatide and globotriaosylceramide are all abnormally increased. In fact, the degree of the lactosylceramide accumulation in the liver is again the highest we have ever encountered in any disease, human or animals. From the tissue analyses, no clear GM3-ganglioside accumulation could be demonstrated. However, the serine-loading experiments with cultured fibroblasts indicated that degradation of GM3-ganglioside might also be impaired, consistent with the finding of Fingerhut *et al.*, (65) on stimulation of GM3-ganglioside by SAP-B *in vitro*. This apparent discrepancy might be due to the nearly absent GM3-ganglioside in the mouse liver. On the other hand, there was no evidence for any abnormal degradation of sphingomyelin despite the fact that stimulation of its degradation has been demonstrated *in vitro* by three of the four SAPs. This simply underscores the caution necessary in interpreting *in vitro* observations.

The increases in polysialogangliosides in the brain are unusual and cannot be readily explained from the known properties of SAPs. The genetic disease that occurs among the emu appears to be the only condition in which a similar abnormal brain ganglioside pattern is known (37). In fact, the glycolipid compositions of the brain and liver of these birds in a more recent detailed study were reminiscent of the human and murine total SAP deficiency states (66). While nothing is known about SAPs in birds, the disease among the emu may well be a naturally occurring animal model of total SAP deficiency.

Activities of lysosomal enzymes were determined in the liver with the rationale that changes in the enzyme activities secondary to the very severe neuropathology would complicate interpretation of the results in the brain tissue. The only genetic lesion in the affected mice is the inactivation of the SAP gene and we had anticipated essentially normal or elevated activities of lysosomal enzymes as often is the case in such conditions. However, a few clear differences were noted between the affected and control animals. Any alterations must be the consequences of the underlying SAP gene deficiency. The most conspicuous was the 75% decrease in the glucosylceramidase activity, either with the natural or artificial substrate. Among the four SAPs, SAP-C has been suggested to primarily interact with the enzyme, glucosylceramidase, rather than with the substrate, to effect its activator function (67–69). Our observation here suggests that SAP-C, and by implication, other sphingolipid activator proteins, may function also as 'protective proteins' *in vivo*, analogous to the protective protein for the β-galactosidase/α-neuraminidase complex (70). From this perspective, activities of two other enzymes, galactosylceramidase and sphingomyelinase, were also substantially lower in the affected mice. The analytical findings in the kidney suggest that one or more of the SAPs might have the physiological function as the activator or possibly the stabilizer of galactosylceramidase. Both sap-A (50) and sap-C (71) have been shown to activate galactosylceramidase *in vitro*. Three of the four SAPs activate *in vitro* degradation of sphingomyelin (72–74,97). Although none is required for its *in vivo* degradation, as clearly shown by the normal tissue levels of sphingomyelin in the affected mice as well as in the complete sphingolipid activator deficient human patients, one or more of them may function as a stabilizer of sphingomyelinase *in vivo*. It should also be noted that ceramidase activity was also reported to be low in the human patients with total SAP deficiency. On the other hand, three enzymes that are pertinent to SAP-B are either normal or elevated in the affected mice, arylsulfatase A for sulfatide degradation, α-galactosidase for globotriaosylceramide degradation, and acid β-galactosidase for GM1-ganglioside degradation. Degradation of sulfatide and globotriaosylceramide is stimulated by SAP-B *in vivo*, while *in vitro* activation by SAP-B has been reported for GM1-ganglioside (1).

The most important observation on the results of the serine-loading test with cultured fibroblasts is that the abnormalities in the SAP –/– mice are essentially identical to those in the fibroblasts of the human patients with the total SAP deficiency. It should also be noted that tissue accumulation of multiple sphingolipids and reduced activities of glucosylceramidase, galactosylceramidase, and ceramidase were also described in the human disorder (24,26–28). Thus, the functions of the sphingo- lipid activator proteins are similar in the two species and this mouse disorder is an excellent model for the known human disease. Table 3 provides comparison of the total sap deficiency in the two species.

While most of our observations so far appear to be understandable from the known properties of the four sphingolipid activator proteins generated by this gene and this model should be useful for further dissecting out the functions of the individual SAPs, the greatest potential importance of this model lies in the possible exploration of the apparently highly complex physiological functions of the other products of this gene. The question of real identity of the SAP gene and the Sertoli cell major sulfated glycoprotein gene can be answered by careful examination of the testis of the affected male mice. The putative functions of the precursor protein as a ganglioside binding/transport protein or as a neurotrophic factor can be further evaluated with this model. Our initial series of morphological examinations described in this report did not indicate obvious abnormalities suggestive of deficiency of an essential neurotrophic factor. However, our study so far must be considered preliminary in this regard and must be expanded with more sophisticated morphometric studies. O'Brien *et al.* (33) have proposed a specific 11-amino acid stretch within the human SAP-C domain as required and sufficient for the proposed neurotrophic function. The equivalent region of the mouse gene can be manipulated and transgenically introduced into these SAP –/– mice which do not produce the endogenous counterpart.

Table 3. Comparison of human and murine total sap deficiency

	Human disease	Murine disease
Molecular genetics		
mutation	ATG → TTG	disruption of exon 3
mRNA	+	−
Clinical features		
rapidly progressive multisystem	+	+
respiratory difficulty	+	?
seizures	+	+
motor disturbance	+	+
contracted hand joints	+	−
organomegaly	+	−
Pathology		
leukodystrophy	? (no autopsy)	++
brain atrophy	+ (by MRI)	−
Gaucher-like cells in liver	+	−
Analytical biochemistry		
ceramide	↑ (L, K)	↑ (L)
glucosylceramide	↑↑ (L)	↑ (L, K)
lactosylceramide	↑↑↑ (L, K)	↑↑↑ (B, L, K)
galactosylceramide	↑ (×2 in K)	↑ (×5 in K)
sulfatide	↑ (×5 in K)	↑ (×5 in K)
GM3-ganglioside	normal (×1.3 in K)	???
polysialogangliosides	???	↑ (B)
Enzymatic activities		
ceramidase	↓↓ (F, AFC)	not determined
glucosylceramidase	↓↓ (F), ↓ (F^a, W)	↓↓ (L)
galactosylceramidase	↓↓ (F, F^a, W), ↓ (L)	↓ (L)
sphingomyelinase	normal (F, F^a, L)	↓ (L)
arylsulfatase A	normal (F)	normal (L)
β-galactosidase	normal (F)	normal (L)
α-galactosidase	normal (F)	normal (L)
Impaired catabolism in fibroblasts	ceramide, glucosylceramide, lactosylceramide and GM3-ganglioside	identical to human disease

B: brain; L: liver; K: kidney; F: cultured fibroblasts; W: leukocytes; AFC: amniotic fluid cells.
[a]Assayed with substrates in liposomes without detergents.

Feeding of the SAP precursor protein to cultured fibroblasts from the human patients at nanomolar concentrations could effectively normalize the abnormal sphingolipid metabolism and cellular morphology (Burkhardt *et al.*, unpublished observation). The observation of Wenger (75) that introduction of SAP cDNA through a retroviral vector into cultured fibroblasts could correct the blocked sulfatide degradation encourages further exploration of gene transfer and expression for eventual gene therapy trials. The general similarity of the mouse model to the human total sphingolipid activator deficiency makes the murine model suitable for studies of the pathogenesis and feasibility of various therapeutic approaches.

MATERIALS AND METHODS

Molecular biology

Cloning of the mouse SAP gene from the 129 strain. The 2.7 kb mouse cDNA in pGEM-4Z encoding the sphingolipid activator proteins (76) was excised from the vector and further digested with *Eco*RI and *Sph*I to 1.0 and 1.7 kb fragments and subcloned into pUC18 (pSAP-S and pSAP-L). The pSAP-S which spanned exons 2 through 10 was used to screen a genomic library from the mouse 129SV strain in λ-FIX II essentially according to the

manufacturer's instructions (Stratagene, La Jolla, CA). Altogether 11 clones were isolated. One of the clones, designated as λ20–1, spanned 15 kb and included exons 2 through 15.

Targeted disruption of the endogenous gene. The overall strategy for the targeting was to disrupt exon 3 with the neomycin-resistant gene (Fig. 1). Culture of the BK4 cells, a subclone of E14G2a derived from strain 129/Ola mice, electroporation of the *Not*I-linearized targeting vector and screening for targeted ES cells by double selection with G418 and Ganciclovir were done essentially as previously described (77–79). Southern blot analysis was used for screening for the targeted disruption of the SAP gene in the ES cells. Genomic DNA was digested with either *Hin*dIII or *Bam*HI and a 1.7 kb fragment of the gene that included exon 2 was used as the probe (Fig. 1). Two positive clones were identified out of 74 screened. They were micoplasm-free and had the karyotype of 60% (S-25) and 100% (S-29), respectively. Cells from the S-29 line were injected into blastocysts. Germline transmitters among the resultant chimeric mice were mated to generate heterozygous mice and subsequently homozygous mice for the disrupted SAP gene.

Northern analysis. Northern blot analysis was done by the standard procedures with total cellular RNA isolated with the Trizol LS reagent (Gibco-BRL) either from the whole body of neonatal pups or from the livers of older mice. A subclone of λ20–1 spanning exons 2–10 was used as the probe. A 28S rRNA-specific oligonucleotide was end-labelled with [γ-^{32}P]ATP and used as the probe for the internal control for the quantity of RNA applied to the gel.

Histopathology

Brain, spinal cord, peripheral nerves and visceral organs from a 30 day old SAP –/– and littermate control mice, and selected tissues (spinal cord, eye, trigeminal and spinal dorsal root ganglions, trigeminal and sciatic nerves) from two 30 day old SAP –/– mice, were examined histologically.

One each of SAP –/– and SAP +/+ mice was anesthetized and perfused with 4% paraformaldehyde. Brains, spinal cord, nerves and various visceral organs were processed for paraffin and plastic embedding. Portions of the cerebrum, cerebellum, spinal cord, eye, dorsal root ganglia and trigeminal and sciatic nerves were cryoprotected by immersion in 0.1 M sodium phosphate buffer (pH 7.4) containing 30% sucrose, quick-frozen in cooled isopentane and sectioned at 7 μm thick with a cryostat. Tissues from two 30 day old SAP –/– mice that were killed for biochemical analysis were immersion-fixed *in situ* for a few days, tissues removed and processed for paraffin embedding.

The paraffin sections of the CNS and PNS tissues were stained with hematoxylin and eosin, solochrome and eosin, luxol fast blue/periodic acid and Schiff (LFB-PAS) and with the Bielschowsky stains. The systemic organs were stained with hematoxylin and eosin, and with periodic acid/Schiff (PAS). In order to evaluate the degree of myelination, paraffin sections of the cerebrum, cerebellum and spinal cord were processed for immunocytochemical detection of the myelin basic protein with the SMI 99 antibody diluted 1:1 000 (Sternberger Monoclonals Inc., Bethesda, MD) using the Autoprobe III kit with streptavidin-conjugated peroxidase base (Biomed Co., Foster City, CA). Frozen sections of the CNS and PNS tissues were stained with PAS, Sudan IV, toluidine blue and acidic cresyl violet.

Plastic-embedded tissues were sectioned in 1 μm thickness and stained with toluidine blue. Selected areas of the cerebrum and liver were further processed for thin sectioning, stained with uranyl acetate and lead citrate and examined with a Zeiss 10A electron microscope.

Analytical biochemistry

Samples. The brain, liver and kidney obtained from four affected and five control mice, all 30 day old, were included in this series of the first characterization. Initial lipid and enzyme analyses were all performed separately on specimens from individual mice. The five control mice included three wild-type and two heterozygous carriers. In no instances were there any differences between the wild-type and heterozygous mice and the results of the control group were treated together for statistical evaluation. For specific analytical purposes, lipid extracts from some samples were pooled at later stages as indicated. Brains were dissected into gray and white matter. Bilateral kidneys were used together for each mouse without further dissection, while appropriate portions of the liver were dissected for the liver samples. The ranges of the samples used for analyses were 54–157 mg (gray matter), 38–92 mg (white matter), 361–490 mg (liver) and 166–286 mg (kidneys).

General lipid extraction. The initial extraction was based on the method of Svennerholm and Fredman (80) as described in detail by Svennerholm *et al.* (81). The tissue was homogenized with 0.6 ml of water in an all-glass Potter-Elvehjem homogenizer and extracted by addition of 2 ml of methanol and 1 ml of chloroform. After at least 15 min, the extract was centrifuged at 1 000 *g* for 10 min and the supernate removed to a small round bottom flask. The pellet was resuspended in 0.7 ml water and re-extracted with 3 ml of chloroform-methanol, 1:2 (v/v). After centrifugation, the combined supernates were taken to dryness in a rotary evaporator and the lipids dissolved in 5 ml of chloroform-methanol-water (60:30:4.5, v/v/v). The extract was desalted through a 1 g Sephadex G-25 Fine column (82).

Separation of gangliosides from other lipids. Gangliosides were separated from other lipids on a 1 g silica gel column (Silica gel 60, 230–400 mesh, Merck A.G., Darmstadt, FRG) packed in chloroform (Svennerholm *et al.* 1991). The lipid sample was redissolved in 2 ml of chloroform-methanol (9:1, v/v), applied to the column, the flask rinsed and applied to the column with two portions of 1 ml of the same solvent. The flask was finally rinsed with 2 ml of chloroform-methanol-water (65:25:4, v/v/v) which was also added to the column. Finally, the column was eluted with additional 6 ml of chloroform-methanol-water (65:25:4, v/v/v). All the eluates to this point were pooled and designated as the 'non-ganglioside lipid fraction'. Then the 'ganglioside fraction' was eluted with additional 10 ml of chloroform-methanol-water (3:6:2, v/v/v). Test runs and thin-layer chromatography indicated that this procedure cleanly separated all non-ganglioside lipids including sphingomyelin into the first fraction and all gangliosides including GM3 into the ganglioside fraction without cross-contamination.

Removal of glycerophospholipids from the non-ganglioside lipid fraction. Aliquots of the brain 'non-ganglioside lipid fraction' were subjected to the mercuric chloride-saponification procedure essentially according to Abramson *et al.* (83). After the alkaline

hydrolysis step, the solutes were partitioned, the upper phase discarded and the lower phase washed twice more. The final lower phase was evaporated to dryness and dissolved in a known volume of chloroform-methanol-water (60:30:4.5, v/v/v). All glycerolipids including plasmalogens abundant in the brain were completely degraded and removed by this procedure, leaving cholesterol, cholesteryl esters, liberated free fatty acids, and sphingolipids. The mercuric chloride step was omitted for the liver and kidney lipids since they contain little plasmalogens.

Separation of kidney sulfatide and GM3-ganglioside. Pooled aliquots from kidney lipids (corresponding to 100 mg tissue from each mouse) were used to evaluate specifically the state of monohexosylceramides, sulfatides and GM3-ganglioside. Sulfatides and gangliosides were isolated by anion-exchange chromatography on a 0.1 g column of DEAE-Sephadex A-25 in acetate form (84) packed in C-M-W (30:60:5, v/v/v). The sample was dissolved in 2 ml of the same solvent, applied to the column and washed with 6 ml of the same solvent. This pass-through fraction contained all of the neutral sphingolipids. Acidic lipids were eluted with 8 ml of C-M-0.8 M potassium acetate (30:60:5, v/v/v). This fraction containing sulfatide and gangliosides was saponified and desalted on a Sephadex G-25 column as described above.

Quantitation of lipids. The total lipid phosphorus, sphingomyelin and cholesterol were determined on the 'non-ganglioside lipid fraction' as previously described (85). The total sialic acid content of the ganglioside fraction was determined by the resorcinol method (86,87) with *N*-acetylneuraminic acid as the standard. Thin-layer chromatography was done with the high-performance thin-layer plates (HPTLC 60, Merck) developed in appropriate solvents: double-development in chloroform-methanol-water (65:25:4, v/v/v) followed by hexane-ethylacetate-acetic acid (90:10:1,v/v/v) for general lipid visualization, chloroform-methanol-acetic acid (47:1:2, v/v/v) for ceramide, and chloroform-methanol-0.2% $CaCl_2$ (55:45:10, v/v/v) for gangliosides. Glucosylceramide and galactosylceramide were separated on borate-impregnated thin-layer plates (88). Lipids were visualized by copper acetate-phosphoric acid (general), orcinol-sulfuric acid (carbohydrate) or resorcinol (gangliosides) (86) sprays and heating. For quantitation, samples were applied with a CAMAG Linomat IV apparatus together with appropriate calibrated lipid standards and densitometry of the plates performed using a CAMAG TLC scanner model II operated with the PC-CATS 3 evaluation software. Special care was taken to keep densities of the bands within the linear range with respect to the quantity of the lipid. For this series of initial characterization, lipids were identified primarily by their TLC mobility compared with authentic lipid standards prepared in the laboratory from normal and pathological human tissues. Because of the small sizes of the starting specimens and of the multiple analytical steps, the recovery of glycolipids present in small quantities are not expected to be quantitative. However, such losses during the analytical procedures should be no more than 30% for the brain and liver samples and should be consistent among all samples. Even in the kidney, where the loss can be even greater, the expected consistent losses among samples allow meaningful comparison between the affected mice and the controls. Recoveries of the major lipids, such as cholesterol, total phospholipids, sphingomyelin and gangliosides should be essentially quantitative.

Enzyme assays

Assays for activities of the lysosomal enzymes were done with the liver from the same mouse specimens as used for lipid analyses. Total homogenates (2–4 mg protein/ml) prepared in ice-cold distilled water and subjected to 3 cycles of freeze-thawing/1 min sonication in a water bath-type sonicator (Branson Ultrasonic Corp., Danbury, CT) were used as the enzyme source. Activities of total β-hexosaminidase, β-galactosidase, β-glucosidase and α-galactosidase were assayed with fluorogenic 4-methylumbelliferyl substrates according to the procedures routinely used for diagnosis of patients with sphingolipidoses (89). The assay system for β-glucosidase with 4-methylumbelliferyl β-glucoside as the substrate included sodium taurocholate to measure specifically that portion of β-glucosidase that represents glucosylceramidase (90,91). Micromethods with radioactively labelled natural lipid substrates were used for assays of glucosylceramidase, acid sphingomyelinase and galactosylceramidase (89,92,93). Arylsulfatase A activity was determined with p-nitrocatechol sulfate on the pH 5.0 supernatant at 0°C to minimize the interference from arylsulfatases B and C (94). The content of protein was determined by a modified Lowry procedure with bovine serum albumin as the standard (95).

Metabolic studies with cultured fibroblasts

Fibroblast cultures were established from 30 day old wild-type and affected mice with the standard procedure of our laboratory. The life span of mouse fibroblasts was relatively limited when the culture was started at such a late age. Cells tended to divide rather slowly and then remained quiescent after several divisions. Normal human fibroblasts were from the Johanniter Children's Hospital, Sankt Augustin, Germany. The SAP-precursor-deficient fibroblasts from the patient who died at 16 weeks were kindly provided by Prof. Harzer (Universität Tübingen, Germany), and have been described before (24,28,29). Cells were cultured as described by Weitz *et al.* (96). The metabolic studies were performed with confluent cells in 21 cm^2 plastic culture dishes. Sphingolipids of cultivated fibroblasts were labeled with L-[3–^{14}C]serine for 24 h, chased for 120 h, extracted and their radioactivity determined after thin-layer chromatographic separation essentially as described previously (51).

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